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CHEMICAL, ENZYMOLOGICAL AND PERMEABILITY PROPERTIES OF HUMAN ERYTHROCYTE GHOSTS PREPARED BY HYPOTONIC LYSIS IN MEDIA OF DIFFERENT OSMOLARITIES

T. A. BRAMLEY*, R. COLEMAN AND J. B. FINEAN

Department of Biochemistry, University of Birmingham, Birmingham, B15 2TT (Great Britain)

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SUMMARY

1. Human erythrocyte ghosts were prepared by controlled hypotonic lysis in a series of bicarbonate buffers of various osmolarities at pH 7.4.

2. Ghosts prepared at moderate osmolarities retain haemoglobin and intracellular enzymes and show low apparent Mg^{2+}-, (Na^+,K^+)- and (Ca^{2+},Mg^{2+})-ATPase activities. The low ATPase activities are principally due to impermeability of the preparation to ATP. Latent ATPase can be demonstrated following short periods of sonication or treatment with detergent.

3. Ghosts prepared at low osmolarities (below about 20 imosM) have lost more haemoglobin and intracellular enzymes. They show high apparent Mg^{2+}- and (Na^+, K^+)-ATPase activities which are not increased by sonication or detergent treatment. Permeability of the preparation to ATP is high. In spite of this high permeability to substrate no (Ca^{2+},Mg^{2+})-ATPase activity can be demonstrated.

4. On storage at very low osmolarities there is also a loss of acetylcholinesterase activity and the ghosts readily fragment. Both these changes can, however, be minimized by storage of the ghosts in isotonic buffer.

5. The transition from the impermeable to the permeable type of preparation takes place over a fairly small range of osmolarities. In this range (Ca^{2+},Mg^{2+})-ATPase is lost or destroyed and there is a pronounced specific loss of non-haemoglobin protein.

INTRODUCTION

In referring the composition and properties of isolated membrane preparations to those of the same membrane in its original state, it is necessary that the membrane has been isolated free of contaminating material, and studied in a state in which the original properties have been preserved.

The membrane preparation which should present fewest problems in this respect is probably that of the mammalian erythrocyte, a cell which consists simply of a limiting membrane and a membrane-free cytosol. Erythrocyte membrane preparations have been made using hypotonic lysis, fragmentation (sonication or homogenization in

* Present address: Department of Clinical Endocrinology, Birmingham and Midland Hospital for Women, Birmingham, B11 4HL, Great Britain.

isotonic or hypotonic media) or chemical lysis (by detergents or other chaotropic agents). Such techniques have also been applied in the preparation of membranes from many other cells with more complex structures and the experience gained from the erythrocyte membrane studies has a wider relevance to the general problem of the preparation of cell membranes.

It has long been recognized that preparations of erythrocyte membranes have widely variable characteristics, notably with respect to their content of haemoglobin and to their apparent ATPase activities. PONDER[1] came to the conclusion that "There are almost as many kinds of ghosts as there are methods by which they are prepared".

The work of DODGE *et al.*[2] and MITCHELL *et al.*[3] has aided considerably in the understanding of the way in which the chemical and enzymological composition of erythrocyte membranes isolated in media of low osmotic strength varies with changes in the conditions of preparation.

Using a different approach HOFFMANN[4] and HOFFMANN *et al.*[5] have sought to obtain ghosts which could be resealed so as to restore their impermeability to substrates and ions. They could thus be made to entrap an artificial bathing medium to provide a system which could be usefully exploited in the study of transport phenomena (see WHITTAM[6]). Such preparations differ in chemical composition from those normally selected for detailed chemical analysis: similarly the enzymological and permeability characteristics of the membranes used for chemical analysis have not been studied in detail. Different investigators have studied a range of membrane characteristics using a wide variety of erythrocyte membrane preparative techniques so that variations in chemical, enzymological and permeability properties are difficult to integrate.

The present work represents an attempt to understand membrane associated phenomena more fully by following several of the properties of erythrocyte membrane preparations under systematically controlled conditions of haemolysis and storage.

MATERIALS AND METHODS

Chemicals

ATP, acetylthiocholine iodide, L-leucyl-β-naphthylamide hydrochloride, *N*-(1-naphthyl) ethylene diamine dihydrochloride, *p*-nitrophenyl phosphate, 5',5'-dithiobis-(2-nitrobenzoic acid), and haemoglobin were obtained from Sigma Chemical Co., London.

62C47 was a gift of Dr. H. T. Openshaw, The Wellcome Research Laboratories, Kent. [γ-^{32}P]ATP was a gift of Dr. E. Lapetina.

Erythrocytes

Only O^+ cells or blood were used in these studies, and were obtained from a local blood bank. The samples had been taken into acid–citrate–dextrose solution and kept at 0–4°. All samples were used as fresh as possible, usually less than 4 days and never more than 3 weeks after collection. The blood used in these experiments was normal and was usually blood which was unsuitable for transfusion for minor technical reasons.

Preparation of ghosts

The packed cells were twice washed with 4 vol. of ice cold 0.155 M sodium bicarbonate buffer (pH 7.4), and resuspended to give a cell/buffer ratio of 1:1. The

method of preparation of ghosts was based on that of DODGE *et al.*[2] except that bicarbonate buffer (pH 7.4) was substituted for phosphate buffer (pH 7.4) and also that the cell/buffer ratio used both for lysis and for washing was 1:11 instead of 1:30 or 1:120. All operations were carried out at 0–4°.

The cells were lysed immediately and then washed in the appropriate strength buffer (0–80 imosM) until the supernatants were haemoglobin-free. This normally took 5–6 washes. The final pellet of ghosts was then suspended as indicated in the legends for each experiment and stored in closed containers.

Choice and use of buffers

Bicarbonate buffer was used in these studies because the phosphate buffer used in the method of Dodge would have severely affected the phosphatase determinations included in the study. Citrate buffer was avoided because of possible chelation effects on intracellular ions after lysis. Tris–HCl buffers are known to alter their buffering capacity and osmolarity markedly at low ionic strength. Bicarbonate buffer was therefore chosen as the best compromise and was found to maintain constant pH over the period of preparation even at the lowest dilutions. Isotonic sodium bicarbonate (0.155 M) was adjusted to pH 7.4 with HCl at 0°. This solution was diluted with appropriate amounts of water at 0° as necessary to give a solution of the required osmolarity, and the pH adjusted to pH 7.4 with dilute HCl if necessary. All buffers were prepared immediately prior to use and kept in sealed containers.

The osmolarities quoted in this study are ideal osmolarities assuming complete dissociation of sodium bicarbonate into two ions. Adjustment of pH with dilute HCl has been ignored since the addition of HCl causes production of H_2CO_3 which partially compensates for the added ionic species. The osmolarities used will not represent precisely the true osmolarities since the behaviour of the ionic species is not ideal.

Protein determinations

An aliquot of the ghost suspension was accurately diluted with 0.1 M KOH and allowed to stand overnight to give a homogeneous solution. Aliquots of this solution were then taken for estimation of protein and haemoglobin.

Protein was determined by the method of LOWRY *et al.*[7] using bovine serum albumin as standard.

Haemoglobin

This was estimated by the pyridine haemochromagen method described by DODGE *et al.*[2] using solutions of crystalline beef haemoglobin as standard.

Non-haemoglobin protein

This figure was obtained by subtracting the value obtained for haemoglobin from that obtained for total protein.

Lipid extraction

The method of BLIGH AND DYER[8] was used but with 2 M KCl in place of water[9]. The extracts were analysed for phospholipid and cholesterol[10].

Phosphate determinations

Inorganic phosphate was determined by the method of FISKE AND SUBBAROW[11].

Organic phosphate was determined by the method of KING[12]. For the determination of phospholipid content, organic phosphate was measured on the lipid extracts. As the content of phospholipid phosphorus in a lipid extract was equal to the total organic phosphate of the appropriate ghosts, determinations of organic phosphate in the ghosts were occasionally used to determine phospholipid content.

Expression of results and standardisation of values

The protein content of membranes could not be used as a reference criterion because of the variations in protein content as a consequence of the preparative conditions (see Fig. 1d). Under these conditions however, phospholipid and cholesterol levels remained constant, in keeping with their roles in membrane composition and structure. All phenomena were therefore related to phospholipid as a measure of the membrane content. When the same suspension of ghosts was used for several studies within a particular experiment, phenomena are reported relative to volume of membrane suspensions.

Enzyme assays

All assays were performed in duplicate or triplicate with appropriate blanks and controls. All assays were carried out at 37° and all activities refer to this temperature.

Acid phosphatase (EC 3.1.3.2) was assayed by a modification of the method of VALENTINE *et al.*[13] using *p*-nitrophenyl phosphate in the presence of 0.1 mM ouabain. Acetylcholinesterase (EC 3.1.1.7) was measured as acetylthiocholinesterase by an adaptation[14] of the method of ELLMAN *et al.*[15]. L-Leucyl-β-naphthylamidase was assayed by a slight modification of the method of HUBSCHER *et al.*[16]. (Na^+,K^+)-ATPase and Mg^{2+}-ATPase (EC 3.6.1.3) were assayed by the method of DUNHAM AND GLYNN[17]. (Ca^{2+},Mg^{2+})-ATPase (EC 3.6.1.3) was assayed by the method of WINS AND SCHOFFENIELS[18].

ATPase assays in the presence of Triton X-100

The detergent caused a turbidity which interfered with colour measurement. The following modification was therefore employed: to 1 ml of assay medium was added 3.75 ml of methanol and the colour developed with 0.5 ml of 72 % perchloric acid, 0.5 ml of 5 % ammonium molybdate and 0.25 ml aminonaphtholsulphonic acid. Full colour development required 20 min: 2 ml of chloroform was then added, and the tubes were shaken and centrifuged. Protein precipitated at the interface and the optical density of the methanol–water upper layer was then measured at 660 nm. The method required internal standardisation with known amounts of inorganic phosphate to allow for changes in extinction coefficient in methanol–water containing Triton, and to account for small losses into the chloroform layer.

Electron microscopy

A drop of buffered osmium tetroxide was added to a drop of ghost suspension deposited on a carbon-covered specimen grid. After 2 min the drop was largely removed and replaced immediately by a drop of distilled water. The drop was replaced several times before allowing the sample to dry completely. The fixed membranes were then examined directly in the electron microscope.

RESULTS

Retention of haemoglobin, acid phosphatase and naphthylamidase

Haemoglobin retention after haemolysis in hypotonic bicarbonate buffers (pH 7.4) is shown in Fig. 1a. This pattern is essentially similar to that observed by DODGE *et al.*[2]. The pattern of retention of acid *p*-nitrophenylphosphatase (Fig. 1b) closely resembles that of haemoglobin. Naphthylamidase retention (Fig. 1c) bears a general resemblance to haemoglobin retention but shows differences in detail. Control experiments demonstrated that latency phenomena were either absent or very low ($< 20\%$) during the assay of these enzymes.

Ghosts prepared in 80 imosM buffer and re-lysed in 10 imosM buffer released all three components in a parallel and recoverable fashion (Table I). Since these two enzymes behave in a similar manner to haemoglobin it is most likely that these activities are located intracellularly.

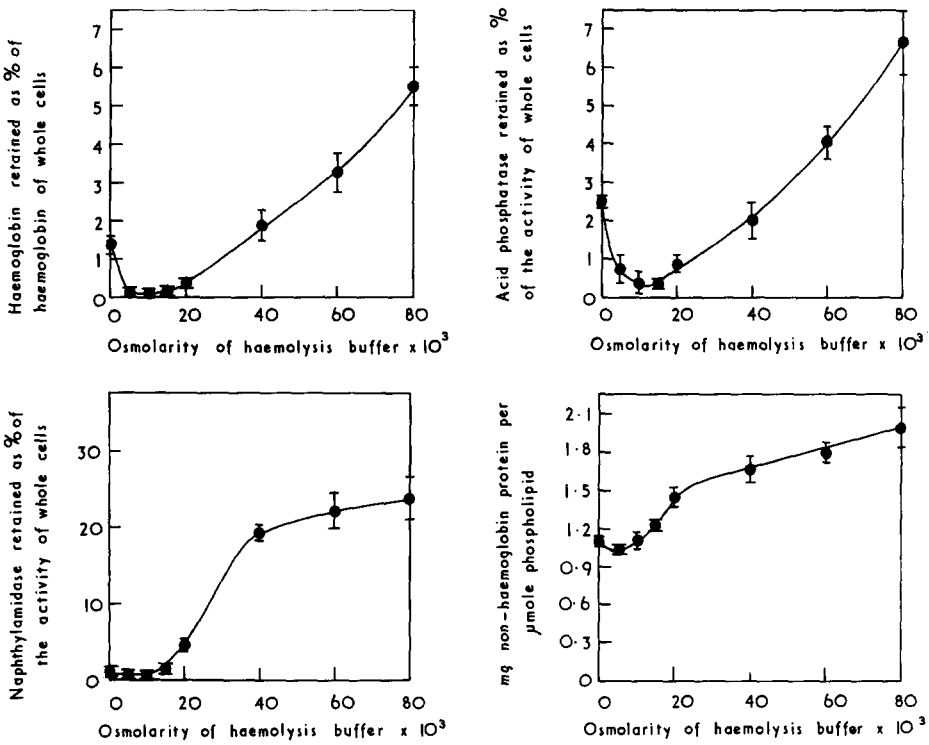


Fig. 1. Composition of human erythrocyte ghost preparations in relation to osmolarity of haemolysis buffer. Preparation conditions and assay of components as in MATERIALS AND METHODS. Ghosts were stored overnight at 0° in the appropriate preparative buffer. Control experiments showed that latency phenomena during assay were absent (naphthylamidase) or less than 20% (phosphatase). Points represent means of several experiments as indicated. Vertical bars indicate the standard deviation. (a) Haemoglobin (10 experiments). (b) Acid *p*-nitrophenylphosphatase (5 experiments). (c) L-Leucyl-β-naphthylamidase (3 experiments). (d) Non-haemoglobin protein (4 experiments).

Non-haemoglobin protein

The pattern of retention of non-haemoglobin protein by ghosts prepared under

TABLE I

RETENTION OF MATERIALS AFTER RE-LYSIS OF ERYTHROCYTE GHOSTS IN A MORE HYPOTONIC SOLUTION

1 ml of ghosts prepared by haemolysis and washing in 80 imosM bicarbonate buffer (pH 7.4) were treated with 4 ml of 10 imosM bicarbonate buffer (pH 7.4) at 0° for 30 min and then centrifuged at 85000 × g_{av} for 30 min. The supernatants were carefully removed and the sediment re-suspended in isotonic bicarbonate buffer (pH 7.4). Supernatant, sediment and original ghost suspension were then examined for appropriate components after storage at 0° overnight. The figures quoted are the means of 3 experiments ± S.E. The figures in parentheses represent the range.

	Original ghost preparation	*Sediment after treatment*	*Supernatant after treatment*	*Recovery* (%)
Haemoglobin (mg)	2.73 ± 0.03	0.46 ± 0.04	2.29 ± 0.07	100 (96–105)
L-Leucyl-β-naphthylamidase (nmoles/h)	50.5 ± 0.6	10.6 ± 0.2	41.3 ± 0.1	103 (100–105)
Acid *p*-nitrophenylphosphatase (nmoles/h)	144.0 ± 7.0	36.0 ± 3.0	114.0 ± 3.0	104 (98–114)

conditions of controlled osmolarity was more complex than that of haemoglobin (Fig. 1d). In addition to the slight fall in protein between 80 and 20 imosM which is probably due to a loss of intracellular proteins, there was a sharp and reproducible fall in the protein content of ghosts prepared below 20 imosM. This pattern was not observed by MITCHELL *et al.*[3] who did not study non-haemoglobin protein in such detail.

It will be shown that the membranes of ghosts prepared at low osmolarities appear to be more susceptible to damage than the membranes of ghosts prepared at higher osmolarities, and that this lability may be associated with the loss of non-haemoglobin protein.

Acetylcholinesterase and morphology

The acetylcholinesterase contents of ghosts prepared and stored in the appropriate haemolysing solutions are shown in Fig. 2a. Ghosts prepared in buffers greater than 20 imosM retained all of the cholinesterase of the intact cells, but there was a progressive loss of cholinesterase activity below 20 imosM. No cholinesterase activity could be detected in any of the ghost-free haemolysates or subsequent wash solutions.

Exposure of ghosts prepared at 20 imosM to a medium of lower osmolarity caused a loss of cholinesterase activity which could not be accounted for in the supernatant (Table II). Under the conditions used in Table II, recoveries of acid phosphatase and naphthylamidase were quantitative. Thus there appears to be a progressive destruction of acetylcholinesterase activity in ghosts during storage in buffers of low osmolarity. This lability was paralleled by a progressive fragmentation of the ghosts as seen by electron microscopy (Figs. 3a–3c) and phase contrast microscopy. Ghost suspensions stored overnight at low ionic strength showed a slow rise in pH from 7.4 to 8.1 and it is possible that the combination of low ionic strength and elevated pH was responsible both for the loss of cholinesterase activity and the morphological damage. In these experiments the phenomena were observed in dilute bi-

carbonate buffers, but they are also evident in human erythrocyte ghosts stored in other very dilute buffers (see ref. 2).

These changes of cholinesterase activity and morphology were minimal in freshly prepared material and developed during storage in dilute buffers. They could, however, be largely prevented by storage in isotonic buffer (see Figs. 2b and 3d), or by the addition of small amounts of Ca^{2+} (ref. 27).

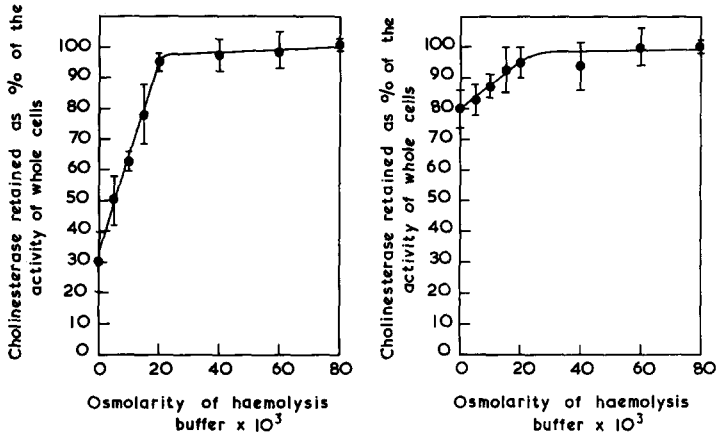


Fig. 2. Acetylthiocholinesterase activity of ghost preparations in relation to osmolarity of haemolysing buffer. Preparation and assay described in MATERIALS AND METHODS. (a) Ghosts stored overnight at 0° in appropriate haemolysis buffers (5 experiments). (b) Ghosts stored overnight at 0° in isotonic bicarbonate buffer, pH 7.4 (6 experiments).

TABLE II

LOSS OF ACETYLTHIOCHOLINESTERASE ACTIVITY ON EXPOSURE TO VERY LOW IONIC STRENGTH AFTER PREPARATION

1 ml of a ghost preparation prepared in 20 imosM was treated with 5 ml of distilled water (buffered to pH 7.4 with a trace of sodium bicarbonate) at 0° for 30 min. The mixture was then centrifuged at 85000 × g_{av} for 30 min and the original ghosts, supernatant and sediment assayed for activity (experiment performed in triplicate). The figures quoted are the mean ± S.E. The figures in parentheses represent the range.

	Original ghosts	*Sediment*	*Supernatant*	*Recovery* (%)
Acetylthiocholinesterase activity (μmoles/total sample per 10 min)	284 ± 5	160 ± 4	0	56 (55–59

Adenosine triphosphatase activities

Preliminary measurements of the Mg^{2+}- and (Na^+,K^+)-ATPase activities of ghosts prepared and stored in low osmolarity buffers were very variable and no consistent phenomena were observed. Upon storage of the ghosts in isotonic buffers (bicarbonate or Tris–HCl at pH 7.4) this variability disappeared.

In contrast to the phenomena described above for other components, the ATPase activities of the ghosts showed a progressive increase as the osmolarity of the haemolysis buffer was reduced (Fig. 4a). Both Mg^{2+}- and (Na^+,K^+)-ATPase activities behaved in a similar fashion. The factors controlling the apparent ATPase activity behave in an exponential fashion (Fig. 4b).

In order for ATPase activity to be shown, the substrate must have access to the substrate sites (active centre) on the interior of the membrane[6]. Thus the observed pattern of ATPase activities could be explained if lysis in moderate osmolarity buffers

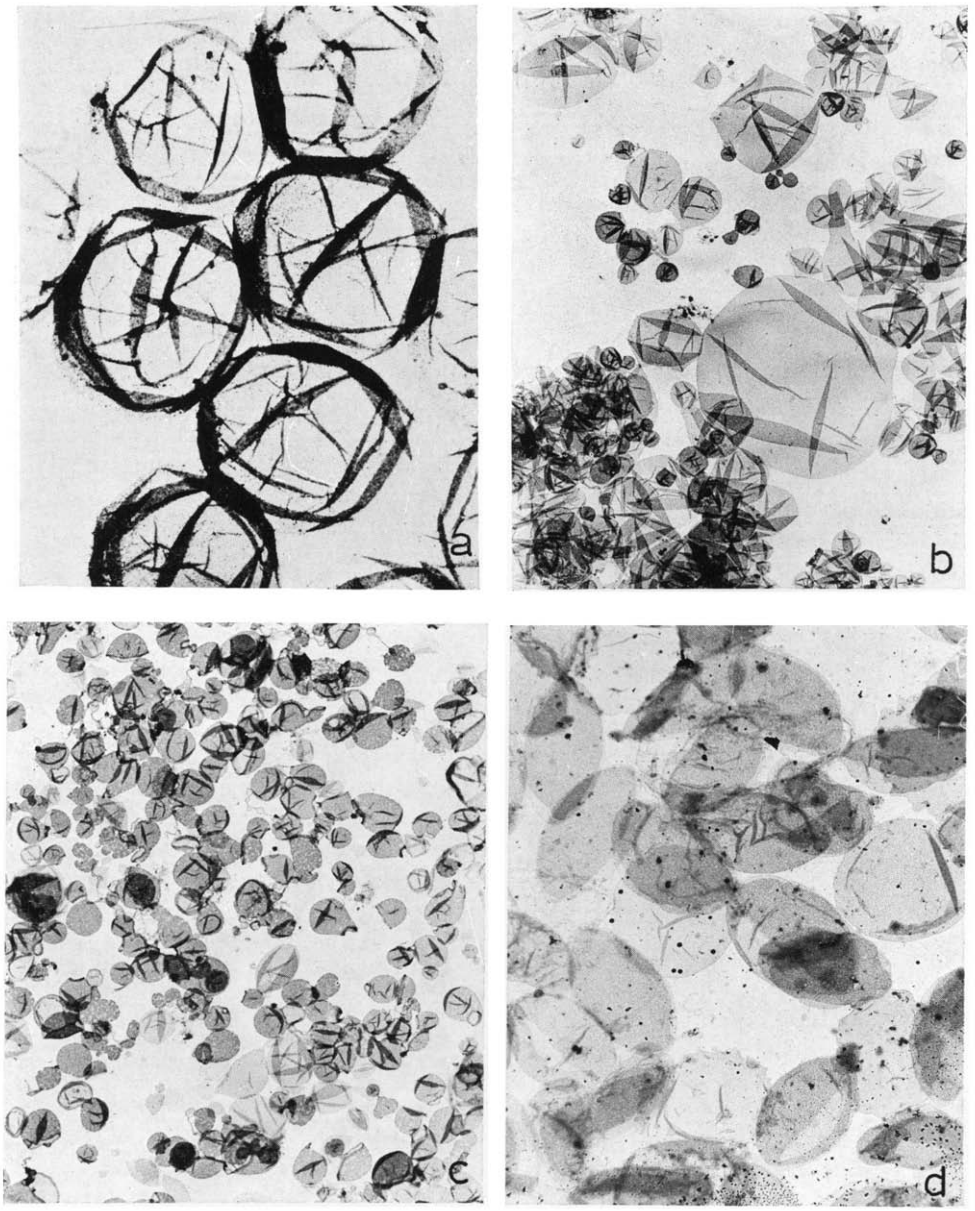


Fig. 3. Electron micrographs of human erythrocyte ghosts prepared and stored under a variety of conditions. Ghosts fixed with OsO_4 in suspension and dried down on carbon film. Magnification approx. × 1500. (a) Ghosts prepared and stored in 15 imosM bicarbonate buffer (pH 7.4). (b) Ghosts prepared and stored in 5 imosM bicarbonate buffer (pH 7.4). (c) Ghosts prepared and stored in distilled water pH 7.4. (d) Ghosts prepared in distilled water and stored in 0.155 M bicarbonate buffer (pH 7.4).

had produced ghosts with low permeability to ATP whilst lysis at progressively lower osmolarities had produced ghosts increasingly more permeable to ATP. If this is the case then the ATPase activity of a particular preparation of ghosts should depend in part upon the permeability to ATP. An alternative explanation may be a greater leaching out or destruction of the activities at moderate osmolarities than at low osmolarities.

In a few experiments the ATPase activity rose between 60 and 15 imosM and remained constant at this maximal level. This pattern occurred less frequently than the one depicted in Fig. 4b and probably represents the attainment of maximum permeability and fragility somewhat earlier than in the majority of preparations.

From the results in Fig. 4a, ghosts prepared at a moderate osmolarity (80 imosM) may be largely impermeable to ATP, resulting in masking of the maximum potential activity. If, however, the permeability barrier of these ghosts is disrupted, this should result in greater ATP penetration and higher ATPase activities, *i.e.* an "unmasking" of the latent ATPase.

Ghosts preparations prepared at low (10 imosM) osmolarities may be already relatively permeable to ATP and therefore parallel treatment of these should not result in significant "unmasking" of the ATPase activity. The ATPase activities of the various preparations of ghosts were therefore studied under conditions which might be expected to destroy the ATP permeability barrier.

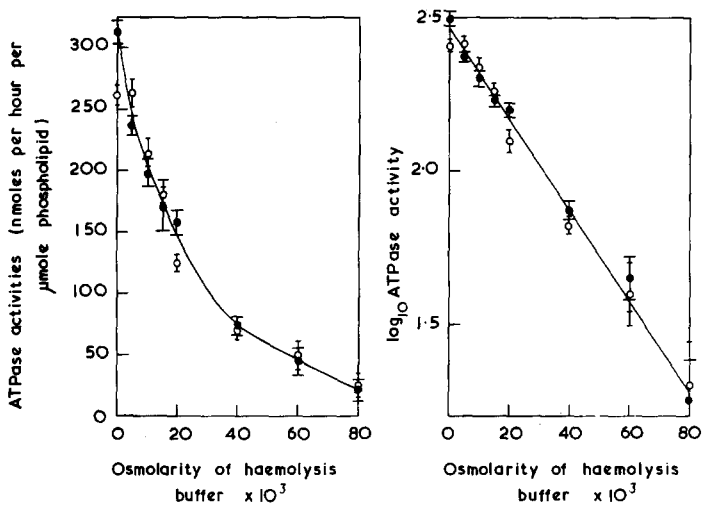


Fig. 4. ATPase activities of ghost preparations in relation to osmolarity of haemolysis buffer Preparation of ghosts in bicarbonate buffer of osmolarity indicated. Each preparation then washed twice in 12 vol. of isotonic Tris–HCl buffer (pH 7.4), resuspended in isotonic Tris–HCl buffer (pH 7.4) and stored overnight at 0° prior to assay. Assays as in MATERIALS AND METHODS. ●, Mg^{2+}-ATPase; ○, (Na^+, K^+)-ATPase. The points represent means of 3 experiments. The bars indicate the standard deviation. (a) Activities expressed in normal terms. (b) Activities expressed by semi-logarithmic plot.

Sonication of ghost preparations

The effects of sonication for various times on ghost preparations prepared at 80 and 10 imosM are shown in Fig. 5. The immediate effect of sonication on the 80

imosM preparation (Fig. 5a) was to induce a dramatic increase in both ATPase activities which was already apparent at the shortest sonication time used. Thus disruption of the permeability barrier to substrate has resulted in higher demonstrable ATPase activity. It would seem therefore that the enzyme activity had been masked rather than destroyed or leached out in these preparations.

Sonication did not induce an increase in the ATPase activities of 10 imosM preparations (Fig. 5b). This suggests that these ghosts are fully permeable to ATP so that disruption of the ghost membrane has no further effect on the permeability to ATP.

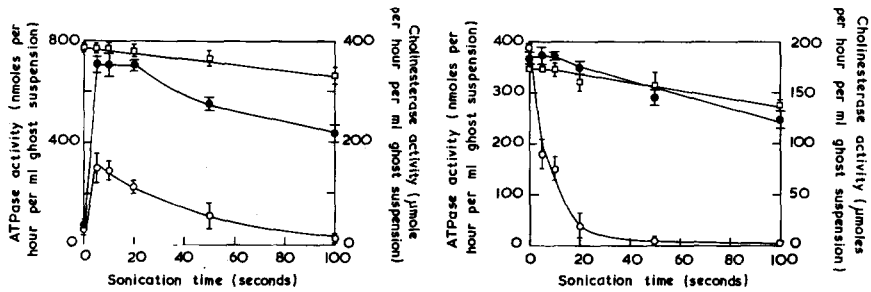


Fig. 5. Effect of sonication on ghost preparations prepared at moderate (80 imosM) and low (10 imosM) osmolarities. Ghosts prepared in the appropriate buffer were washed twice and suspended in isotonic bicarbonate buffer (pH 7.4). Equal volumes of each preparation were subjected to appropriate sonication (M.S.E. sonic vibrator) at 0° for progressive time intervals of 10 sec with a 30-sec cooling interval between each sonication. ●, Mg^{2+}-ATPase; ○, (Na^+,K^+)-ATPase; □, acetylthiocholinesterase. The points represent the mean of 2 experiments, the vertical bars represent the range. (a) Ghosts prepared at 10 imosM. (b) Ghosts prepared at 80 imosM.

In both preparations, acetylcholinesterase was little affected. This enzyme has been suggested to be on the external surface of the membrane[20–22] and therefore would not be subject to permeability limitations for demonstration of full activity.

Further sonication of both preparations brought about a decline in ATPase activities in both cases but most markedly in the case of (Na^+,K^+)-ATPase. This suggests a secondary process had come into operation, either a destruction of the enzyme or the conversion of the membranous material to small vesicles with the re-establishment of permeability limitations. (This point has not been fully resolved. Most subsequent experiments, however, pointed to destruction as the more likely alternative.)

Treatment of ghost preparations with Triton X-100

Fig. 6a shows the effect of a range of concentrations of the detergent Triton X-100 on both the turbidity and Mg^{2+}-ATPase activities of ghosts prepared at moderate osmolarity. Triton had little effect on either parameter at concentrations below 0.01 % then a dramatic fall in turbidity and a marked rise in ATPase activity were observed. The greatest effects were observed in the region of 0.03 %. The fall in turbidity indicated that lysis was occurring in this region since, after centrifugation, haemoglobin was found in the supernatant.

Direct measurement of ATPase activities of ghosts prepared at low osmolarity (10 imosM) in the presence of detergent gave technically unsatisfactory results, and

thus a comparison of the effects of detergent on low osmolarity and moderate osmolarity ghosts was not possible. In this context, however, the effects of detergent treatment on an 80 imosM preparation rendered permeable by a 10-sec sonication are interesting (Fig. 6b). A slight fall in turbidity was observed in the same range of detergent concentrations as with unsonicated preparations but there was no accompanying increase in ATPase activity, and the ATPase activity of the sonicated material declined at higher detergent concentrations. The ATPase activity of the sonicated material was high initially, whilst the low initial activity of the unsonicated material rose to approximately this level after detergent treatment.

The increase in ATPase activity indicated in Fig. 6a could be due either to an activation of the ATPase by detergent or to the removal of permeability limitations. The former is unlikely since no activation of the sonicated material was observed at similar detergent concentrations. The enhancement of ATPase activity shown in Fig. 6a also proves wrong the suggestion that the low activity of the original preparation might be due to the leaching out or destruction of the enzyme. Therefore as long as a permeability barrier is present, disruption (by sonication or detergent) gives a boost in ATPase activity, whereas if the permeability barrier is lost, disruption has no effect.

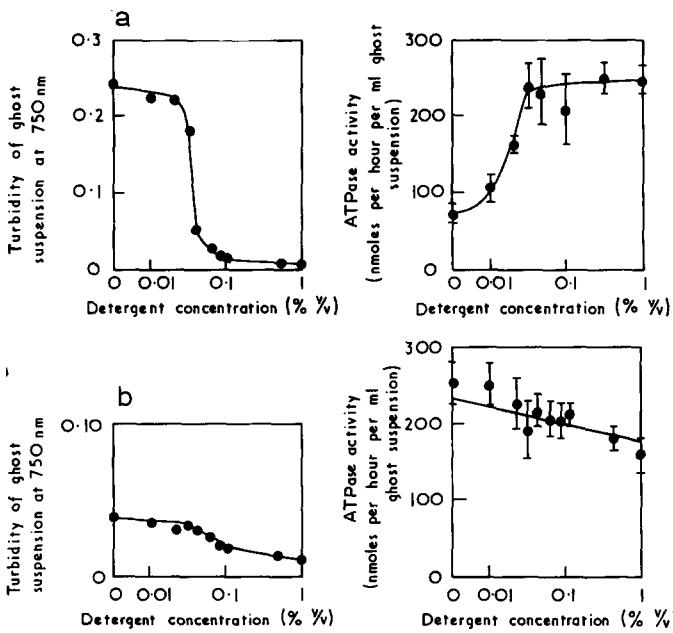


Fig. 6. Effect of Triton X-100 on the turbidity and Mg^{2+}-ATPase activity of ghost preparations before and after sonication. The ghosts were prepared in 80 imosM bicarbonate buffer and washed, resuspended and stored overnight in isotonic bicarbonate buffer (pH 7.4). A portion of the suspension was sonicated for 10 sec at 0°. Samples were assayed for ATPase activity in the presence of the detergent concentrations shown. After removing 0.1 ml for turbidity measurement, the remainder of the assay medium was used for phosphate determination in the presence of methanol as outlined in MATERIALS AND METHODS. For turbidity measurement the aliquots were diluted to 4 ml with isotonic NaCl prior to determination as $A_{750\ nm}$. The points represent a single experiment: the bars represent the ranges of the duplicate determinations. (a) Before sonication. (b) After sonication for 10 sec.

Permeability of ghosts to [^{32}P]ATP

The previous experiments have implicated permeability to ATP as a principal factor controlling measurable ATPase activities. In order to substantiate this hypothesis the permeability of several ghost preparations to ATP was measured directly.

In these experiments the degree of impermeability of the ghosts to ATP was expressed in terms of the percentage of the volume of the ghost suspension inaccessible to ATP. The actual magnitude in any given experiment depended principally upon two factors, (a) the number of ghosts per unit volume and (b) the "space" occupied by each ghost. In order to eliminate (a) suspensions within a given experiment were adjusted to the same phospholipid concentration (a reliable indication of membrane concentration). Thus the principal variable within each set of experiments was the permeability or "apparent space occupied" of a single ghost.

Fig. 7 represents results from a series of experiments in which the relative permeabilities of ghosts prepared at low (10 imosM) and moderate (80 imosM) osmotic strengths were compared with respect to both ATPase activities and impermeability to ATP. It can be seen that ATPase activity and impermeability to ATP bear a striking inverse relationship.

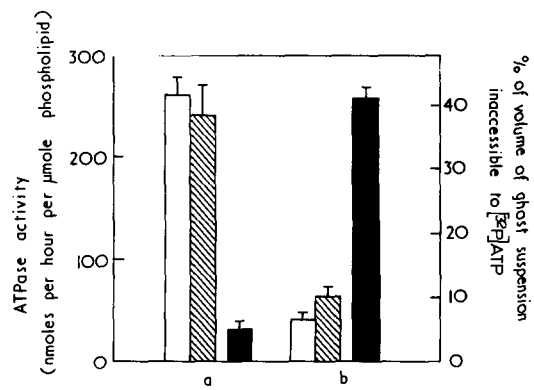


Fig. 7. Relationship of ATPase activities to permeability of ATP in low and moderate osmolarity ghost preparations. The ghosts were prepared by haemolysis and washing in 10 or 80 imosM bicarbonate buffer (pH 7.4) as indicated. Both preparations were washed twice and resuspended in isotonic bicarbonate buffer (pH 7.4) as a concentrated suspension. The preparations were adjusted to the same concentration of phospholipid phosphorus. ATPase activities were measured as described in MATERIALS AND METHODS. To 3 ml of each ghost suspension at 0°, 0.5 ml of [^{32}P]ATP was added, the suspension was mixed, allowed to stand for 5 min at 0° (to minimise ATP hydrolysis), centrifuged at 10000 × g_{av}, 10 min and aliquots of the supernatant removed and diluted for measurement of radioactivity content (liquid Geiger). From these measurements the volume inaccessible to [^{32}P]ATP was calculated. Values given represent means of 4 measurements (ATPase) and 6 measurements (permeability). Bars represent the standard deviation. Open column, Mg^{2+}-ATPase; hatched column, (Na^+, K^+)-ATPase; solid column, impermeability. (a) Ghosts prepared at 10 imosM. (b) Ghosts prepared at 80 imosM.

Measurement of penetration of [^{32}P]ATP into ghosts is subject to the limitation that ATP is also likely to be hydrolysed by the ghosts. The experiments quoted above were conducted rapidly at 0° in order to minimize this effect; the validity of these measurements is supported by comparable values obtained for permeation of ghosts by [^{14}C]sucrose and haemoglobin. These will be discussed in a later communication.

The inaccessibility to [^{32}P]ATP of ghosts prepared at moderate osmolarity

(80 imosM) is largely abolished by sonication for 5 sec. This treatment was sufficient to cause significant increase in ATPase activities (see Fig. 5a).

Effect of osmolarity of the storage buffer upon ATPase activities

From Fig. 4a it appears that the ATPase activity of a preparation of erythrocyte ghosts depends upon the osmolarity of the preparation buffer. In this experiment, all preparations were suspended in isotonic buffer. The osmolarity of the storage buffer also exerted an effect upon the final levels of ATPase activities of the ghost preparation. Storage of ghosts in isotonic (310 imosM) buffer lowered the ATPase activity shown by all preparations compared to storage of the same material in moderate (80 imosM) buffer (Table III). This probably reflects a greater degree of re-sealing (re-establishment of the permeability barrier to substrate) when ghosts are suspended in isotonic buffer as compared to a buffer of lower tonicity. This resealing takes place during storage of the material on ice and does not therefore appear to require incubation at 37° as described by HOFFMAN[19]. Table III also shows that the extent to which isotonic solutions were able to restore the permeability barrier as judged by ATPase levels lessened with decreasing osmolarity of the haemolysing buffer. Ghosts prepared at low osmolarity are therefore less capable of resealing even under isotonic conditions.

TABLE III

ATPase ACTIVITIES OF GHOST PREPARATIONS UNDER DIFFERENT CONDITIONS OF PREPARATION AND STORAGE

Ghosts were prepared by haemolysis and washing in 10, 20, 40, 60, 80 imosM bicarbonate buffers (pH 7.4). The ghosts were then washed and suspended and stored overnight at 0° in either 80 or 310 imosM bicarbonate buffers as indicated. Samples were assayed in duplicate and the figures shown are means.

Haemolysing buffer (imosM):	*10*		*20*		*40*		*60*		*80*	
Storage buffer (imosM):	*80*	*310*	*80*	*310*	*80*	*310*	*80*	*310*	*80*	*310*
Mg^{2+}-ATPase (nmoles/h per μmole phospholipid)	300	283	288	191	220	80	115	63	63	46

(Ca^{2+},Mg^{2+})-ATPase activities in relation to osmolarity of preparation buffer

Fig. 8 demonstrates the behaviour of (Ca^{2+},Mg^{2+})-ATPase in contrast to that of Mg^{2+}-ATPase in the same preparations. Whilst Mg^{2+}-ATPase activity was higher in ghosts prepared at low osmolarities compared to moderate osmolarities, ghosts prepared at osmolarities of less than 15 imosM not only showed no (Ca^{2+},Mg^{2+})-ATPase but also the Ca^{2+} present slightly inhibited the Mg^{2+}-ATPase at this point.

The loss of (Ca^{2+},Mg^{2+})-ATPase activity at preparative osmolarities of below 20 imosM cannot be explained by the lack of penetration of ATP, since under identical conditions of preparation and storage both Mg^{2+}-ATPase and (Na^{+},K^{+})-ATPase (Figs. 4a and 8) showed high, non-latent activities. The absence of (Ca^{2+},Mg^{2+})-ATPase at low osmolarities is also seen under conditions in which permeability barriers have been removed by brief sonication (Fig. 9). This same loss of (Ca^{2+},Mg^{2+})-ATPase at very low osmolarity has been observed when ghosts prepared at 80 imosM were resuspended in low (0–10 imosM) buffers. (Ca^{2+},Mg^{2+})-ATPase was not then ob-

served in the total suspension, sediment, or supernatant after centrifugation. This points to inactivation rather than solubilization under these conditions, though the inactivation may take place during storage of the ghosts.

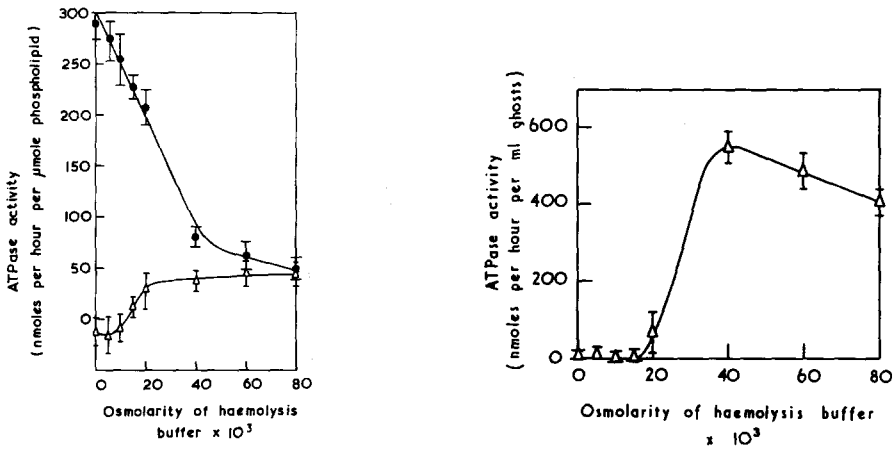


Fig. 8. Mg^{2+}-ATPase and (Ca^{2+},Mg^{2+})-ATPase activities of ghost preparations in relation to osmolarity of haemolysis buffer. Preparation of ghosts in bicarbonate buffer of strength indicated and resuspended and stored as in Fig. 3. Assays as in MATERIALS AND METHODS. ●, Mg^{2+}-ATPase; △, (Ca^{2+},Mg^{2+})-ATPase. The points represent the mean of 2 experiments, the bars indicate the range.

Fig. 9. Effect of sonication on (Ca^{2+},Mg^{2+})-ATPase activities of ghosts prepared in a range of osmolarities. Ghosts were prepared in bicarbonate buffer (pH 7.4) in the range of osmolarities shown, and washed twice, resuspended and stored overnight in isotonic Tris–HCl buffer (pH 7.4) at 0°. Ghosts from the same volume of blood were adjusted to the same final volume. All samples were sonicated for 10 sec at 0° and aliquots examined immediately for (Ca^{2+},Mg^{2+})-ATPase as in MATERIALS AND METHODS. The points represent a single experiment, the bars represent ranges of triplicate determinations.

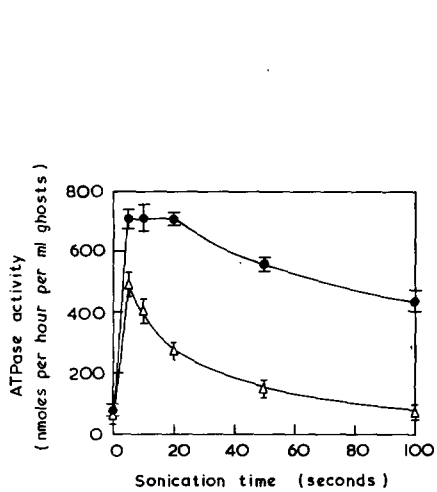
Latency of (Ca^{2+}, Mg^{2+})-ATPase in moderate osmolarity ghosts

When ghosts prepared at various osmolarities are exposed to sufficient sonication to remove the permeability barrier to ATP penetration, (Ca^{2+},Mg^{2+})-ATPase activity is evident (Fig. 10). This is observed in all ghosts prepared at osmolarities above 20 imosM (Fig. 4). These levels are quite high and represent a considerable increase on those shown before sonication (*cf.* Figs. 8 and 9). However, further sonication of moderate osmolarity ghosts leads to rapid inactivation of (Ca^{2+},Mg^{2+})-ATPase (Fig. 10).

When ghosts prepared at moderate osmolarity were treated with increasing concentrations of Triton X-100 (Fig. 11) there was a dramatic increase in the (Ca^{2+}, Mg^{2+})-ATPase activity, the onset of which corresponded with the increase in Mg^{2+}-ATPase activity and the change in turbidity (see Fig. 5a, identical conditions of treatment). The increase in (Ca^{2+},Mg^{2+})-ATPase activity in this region would therefore seem to be related to permeability changes in the preparation. Beyond the point of greatest (Ca^{2+}, Mg^{2+})-ATPase activity, the addition of further detergent appeared to diminish the (Ca^{2+}, Mg^{2+})-ATPase activity, illustrating the ease with which this activity is lost.

Effect of Ca^{2+} in the assay medium in relation to observed (Ca^{2+},Mg^{2+})-ATPase activity

A further complication in the study of (Ca^{2+},Mg^{2+})-ATPase activity in ghost preparations may be the effect of Ca^{2+} present in the assay medium for (Ca^{2+},Mg^{2+})-ATPase upon the apparent permeability properties of the ghost preparation. This is best illustrated by the experiments in Fig. 11. In this experiment increasing the concentration of detergent to the point at which permeability changes were observed (see Figs. 6a and 6b) brought about a dramatic increase in (Ca^{2+},Mg^{2+})-ATPase activity in both cases, after which point the activity of the enzyme rapidly declined due to inhibition or degradation. The sonicated preparation showed a much higher initial level of (Ca^{2+},Mg^{2+})-ATPase (Fig. 11), as might have been expected since sonication appears to remove the permeability limitation for all ATPases studied (Figs. 5a and 10). Detergent, however, produced a further increase in (Ca^{2+},Mg^{2+})-ATPase levels but has no effect on Mg^{2+}-ATPase. This effect could have been due either to a direct stimulatory effect of detergent on the enzyme in the region of critical detergent concentration (0.025–0.075 %) or to an effect upon permeability. The behaviour of the Mg^{2+}-ATPase (Figs. 6a and 6b) at first appears to contradict the latter. The difference between the measurement of the two activities, however, lies in the assay systems. In the assay of (Ca^{2+},Mg^{2+})-ATPase, Ca^{2+} are present in the system and appear to be capable of producing a partial resealing of the sonicated ghost preparation. Further evidence on the resealing of erythrocyte ghosts by incubation in media containing low levels of calcium will be presented in a future communication[27]. The measured final activity of (Ca^{2+},Mg^{2+})ATPase is therefore dependent on both enzymic inactivation during preparation and storage and the final permeability of the preparation resulting from preparation, storage and assay conditions.



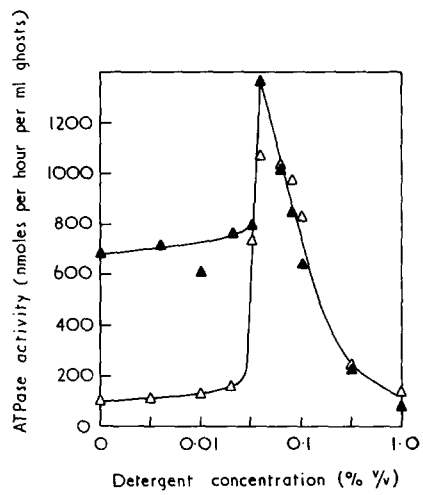


Fig. 10. Effect of increasing sonication time on ATPase activities. For conditions see Fig. 5b. ●, Mg^{2+}-ATPase; △, (Ca^{2+},Mg^{2+})-ATPase. The points represent the means of 2 experiments, the bars represent the range.

Fig. 11. Effect of Triton X-100 on the (Ca^{2+},Mg^{2+})-ATPase activities of ghost preparations before and after sonication. For conditions see Fig. 6. The points represent the average of duplicate determinations. △, before sonication; ▲, after sonication for 10 sec.

DISCUSSION

The work of DODGE *et al.*[2] and MITCHELL *et al.*[3] showed in a systematic manner certain conditions under which the amount of haemoglobin and of other intracellular components retained by erythrocyte ghosts could be controlled. In the present study similar phenomena have been observed for both haemoglobin and two intracellular enzymes, acid *p*-nitrophenylphosphatase and L-leucyl-β-naphthylamidase. The present work shows, in addition, that ghost preparations which retain appreciable amounts of those intracellular components will lyse for a second time in media of lower osmolarity than the original haemolysing buffer. This causes further losses of these intracellular components. The pattern of these losses indicates that these components are probably retained in a manner similar to that in the original erythrocyte. Both of these enzymic activities have been assayed under conditions where measured latency is absent (naphthylamidase) or less than 20 % (phosphatase). The reasons for the slight differences in retention pattern between the two enzymes will be examined in a further communication[27].

The quantitative recovery of cholinesterase relative to erythrocytes in ghosts prepared at moderate osmolarity, and the consistently non-latent nature of this activity in these experiments, is in keeping with its localization as a membrane bound enzyme on the exterior surface of the ghost[20–22].

The observed ATPase activities of ghost preparations depend upon the effects of a number of interacting variables. By altering the preparation and storage conditions and thereby affecting the permeability of the ghost preparation to ATP, the apparent ATPase activities can be controlled with some precision. This control is exerted over a relatively small range in the osmolarity of the haemolysing buffer (see Fig. 3). The principal effects are determined by the osmolarity of the preparation conditions but can be further modified by the osmolarity of the storage conditions (Table III). The ability of the ghosts to reseal after lysis is apparent at 0° and does not require incubation at 37° (ref. 19). Thus ghosts prepared at moderate osmolarities are impermeable not only to the exit of protein molecules during washing but also to the entry of ATP, as evidenced by the low apparent ATPase activities, the latency of ATPase activities and the direct permeability measurements using [γ-^{32}P]ATP, whilst ghosts prepared at low osmolarity are progressively more permeable to ATP and demonstrate high non-latent ATPase activities.

It would seem that the ability of ghosts prepared at low osmolarities to reseal is greatly reduced as compared to ghosts prepared at moderate osmolarities. This occurs under conditions where the highest ATPase activities and permeability are observed. In connection with this, the specific loss of non-haem protein in this same region is especially noteworthy. This loss accounts for approx. 30 % of the non-haem protein that is present in "sealable" ghosts (*i.e.* 40 imosM and above). This loss of protein is considerable and takes place over a narrow range of osmolarity and at physiological pH values. Below 40 imosM only non-haem protein appears to be lost since the phospholipid, cholesterol and sialic acid content of the membrane are unchanged.

In addition to the permeability changes, other effects are evident, notably the total absence of (Ca^{2+},Mg^{2+})-ATPase activity below about 20 imosM (due either to loss or damage). That (Ca^{2+},Mg^{2+})-ATPase is a labile enzyme is shown by its sensitivity to both sonication and detergent. Whilst (Ca^{2+},Mg^{2+})-ATPase is not present below

20 imosM under all circumstances studied, it is present in latent form in ghosts prepared at higher osmolarities and can be demonstrated upon removal of limitations to substrate permeability (Figs. 10 and 11).

These findings suggest that the general permeability and integrity of the membrane are greatly impaired at low osmolarities. Further evidence of this may be the morphological disruption and the loss of cholinesterase activity seen during storage at low osmolarity. These effects can be minimised by the use of isotonic buffers for storage. The region in which the most pronounced changes in membrane properties occurred in the present work was below 20 imosM. In this work minimal haemoglobin retention occurs at 10 imosM. Due to the use of bicarbonate buffer and of a different cell/buffer ratio, this point probably coincides with the minimal retention point observed by DODGE *et al.*[2] at 20 imosM using phosphate buffer. The osmolarity at which minimal protein composition and (probably) maximum permeability occur may therefore vary slightly according to preparative conditions. It is clear from the present studies that further changes in membrane properties occur during storage under conditions of low ionic strength.

There is no direct evidence from the present work to suggest that the dramatic loss of non-haemoglobin protein is directly responsible for the changes in permeability, enzymological or fragility phenomena described above. It is tempting to speculate, however, that erythrocytes possess protein(s), possibly present on the inner surface of the membrane, which has a pronounced effect upon the properties of the membrane and that this protein is released or deactivated at low ionic strengths. This protein may be related to, or contain, some of the other protein components released from erythrocyte membranes[23–26].

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